Characterization of the Collagen–Vinyl Graft Copolymers Prepared by the Ceric Ion Method. I. Solution Properties

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Synopsis

Graft copolymerization of vinyl monomers on to collagen was carried out by the ceric ion method. The grafted vinyl polymer chains were isolated by both acid and enzymatic hydrolysis of the collagen backbone in order to characterize the graft copolymers. Proof of grafting was obtained through the detection of amino acid endgroups in the grafts isolated by both the methods. The grafts isolated gave the characteristic blue color normally associated with the presence of amino acids. The presence of amino acid endgroups was further confirmed by dinitrophenylation of the isolated grafts. The absorption spectra of the dinitrophenylated(DNP) grafts showed absorption maximum in the ultraviolet region of 340–360 mμ, characteristic of DNP-amino acids. Soluble collagen grafted with polyacrylamide(PAA) formed fibrils on heating to 37°C at neutral pH but, unlike the native collagen, these fibrils did not redissolve on cooling at 2°C. These results show that the redispersion property of soluble collagen was impaired, probably by attachment of the PAA side chains to the collagen molecule. The turbidimetric titration behavior of the grafts, their general behavior of swelling in different solvents, and the intrinsic viscosity of the copolymers in mixed solvents also provided additional proof of grafting.

INTRODUCTION

Modification of natural and synthetic polymers by graft copolymerization has been the subject of numerous investigations. However, relatively little effort has been directed toward a systematic investigation of their structure and properties. Quite recently investigations on the structure and properties of the graft copolymers have been taken up by a number of workers.[1–7]

Graft copolymers of collagen and poly(methyl methacrylate)(PMMA) formed through ceric ammonium nitrate(CAN) initiator, have been studied[8–10] in detail to determine what factors govern the percent grafting and the number of grafting sites and the molecular weight of the grafted PMMA. It is common that the graft copolymers are inevitably contaminated with at least one of the homopolymers of the species which make up the copolymer segments. For an unambiguous characterization of these

copolymers, it is necessary to isolate them from the homopolymers present in the polymerization products. The separation poses a major problem when the solubility differences between the graft copolymers and the homopolymers are not large enough to allow adequate separation. Collagen graft copolymers, just as in the case of wool, cellulose, and starch graft copolymers, have the following two advantages from the point of view of composition studies: (1) the solubility difference between the collagen backbone and the grafted side chain polymers is usually sufficiently great to enable adequate separation to be made; (2) the collagen backbone can be hydrolyzed away, thus enabling the molecular weight and the grafting frequency of the grafted side chains to be measured.

This paper is concerned with the physicochemical characterization of collagen–vinyl graft copolymers. The grafted vinyl polymer chains were isolated by both acid and enzymatic hydrolysis of the collagen backbone to characterize the graft copolymers. Several lines of evidence were sought to distinguish between a true graft copolymer and a physical mixture of collagen and vinyl polymers.

EXPERIMENTAL

Materials

Collagen. Collagen prepared from the middle corium of buffalo hide was used as the source of insoluble collagen. Since a uniform stock of material which would be sufficient for the whole investigation was required, a large amount was initially prepared and all the experiments were carried out with the same sample.

Soluble collagen was prepared from calf dermis following a procedure similar to that of Hörmann and Hafter.[11]

Monomers. Methyl methacrylate(MMA) and methyl acrylate(MA) were obtained from Rohm & Hass, U.S.A. and acrylamide was obtained from American Cyanamid Co.

Chemicals. Ceric ammonium nitrate $[Ce(NH_4)_2(NO_3)_6]$ and ceric ammonium sulfate $[Ce(NH_4)_4(SO_4)_4]$ (Puriss, Fluka) were used without further purification. Pronase (B grade, Cal Biochem, U.S.A.) was used without further purification. Other chemicals used were reagent grade.

Purification of Monomers

MMA and MA were purified by washing with a 6–8% sodium hydroxide solution to remove the inhibitor. After this treatment, the monomers were washed with distilled water to remove the alkali completely and dried over anhydrous calcium chloride overnight. The monomers were then distilled under vacuum and stored in a refrigerator. Acrylamide was recrystallized twice with benzene–acetone.

Preparation of Initiator Solution

The required quantity of CAN dissolved in 1*N* nitric acid was used Fresh solutions were prepared for each experiment.

Grafting Procedure

The graft copolymerization reactions were carried out in a round bottomed three-necked flask of one liter capacity fitted with a water-sealed glass st rrer, a gas outlet, and a thermometer. All the grafting experiments were carried out at a stirring speed of 100–150 rpm at room temperature. In a typical experiment, 10 g of hide powder was dispersed in 400 ml distilled water. After oxygen-free nitrogen was bubbled through the solution for 30 min, the required amount of monomer was added followed by ceric ammonium nitrate solution (0.1*M*) in 1*N* nitric acid. The final concentration of monomer was 0.5 mole/l. and that of CAN was 2.5×10^{-5} mole/l. The reaction was allowed to proceed for 3 hr, at the end of which the resulting products were separated by filtration, washed with distilled water, and extracted with the appropriate solvents to remove the loosely bound homopolymer.

Homopolymer Extraction

Preliminary studies with the use of the tumbled bottle method and the Soxhlet extraction method to compare the extraction efficiencies showed that the tumbled bottle method is more efficient for the extraction of the homopolymer. Hence in all subsequent studies this method was followed for extraction of homopolymers from the graft copolymers. The graft copolymers were extracted for 72 hr with two changes of fresh solvents at room temperature. In general, extraction of the crude collagen graft copolymers with solvent for the homopolymer removed only traces of the polymer, and hence no systematic investigations were carried out on the estimation of the amounts of these ungrafted homopolymers.

For grafting on skins, an emulsion polymerization technique was followed.[12] The per cent grafting in the final products were determined from the total nitrogen values as reported previously.[10]

Hydrolysis of the Collagen Backbone with Hydrochloric Acid

Acid hydrolysis was carried out by heating the graft copolymer with 6*N* HCl for 18 hr according to the procedure outlined in a previous paper.[10]

Digestion of the Collagen Backbone with Pronase

Samples of collagen–PMMA graft copolymer (samples GC_1 and GC_3, 0.1–0.3 g) were heat-denatured in water and made 0.02*M* in $CaCl_2$, and the pH was adjusted to 8 with 0.1*N* NaOH. A 10-mg portion of pronase dissolved in 0.5 ml of water was then added, together with a drop of toluene to prevent bacterial growth. The mixture was then incubated at 37°C for

18 hr, while the pH was kept constant by addition of 0.01*N* NaOH. The insoluble graft was removed by centrifugation, washed, and dried. A physical mixture of collagen and PMMA was also digested under identical conditions. The physical mixture (1:1 w/w) was prepared by grinding the mixture well in a mortar. The residues left after enzyme digestion were thoroughly washed with hot water, centrifuged, and dried.

Treatment of the Isolated Grafts with Ninhydrin

Approximately 100 mg of the isolated grafts obtained by the above procedures were weighed and placed into test tubes containing 2 ml of distilled water. A 2-ml portion of the ninhydrin reagent of Moore and Stein[13] was added, and the solutions thoroughly mixed by gently shaking the tubes. The tubes were capped and placed in a rigorously boiling water bath for 20 min. The solution was cooled and 5 ml of the diluent (ethanol–water, 1:1 v/v) solution was added and the whole mixed and centrifuged. The absorbence of the supernatant was measured in a Beckman DU spectrophotometer at 570 mμ.

Grafting on Soluble Collagen

For grafting on soluble collagen it was desirable to use a monomer, the homopolymer of which will also remain in solution after grafting. Acrylamide(AA) was thought to be suitable for this purpose, and hence attempts were made to graft AA on soluble collagen.

A 0.1% solution of soluble collagen in 0.1*N* acetic acid was used for this purpose. The grafting conditions were exactly same as in the case of insoluble collagen. After grafting, the viscous solution was exhaustively dialysed against 0.1*N* acetic acid. The fibril-forming property of the grafted collagen was then studied in comparison with an ungrafted soluble collagen solution and also with a physical mixture of collagen and poly(AA).

Fibril Formation

Soluble collagen in cold neutral salt solutions has the interesting property of precipitating on warming to 37°C as a rigid gel composed of fibrils. If the gel is cooled immediately to 2°C, it will redissolve and will form again upon reheating. Conditions which enhance hydrogen bonding favor the formation of fibrils, while those which rupture hydrogen bonds reverse the equilibrium. It was, therefore, of interest to examine the fiber-forming and resolution properties of the grafted collagen solution. Fiber formation was carried out and measured mainly as described by Gross and Kirk.[14]

Redispersion was studied by placing the opaque gelled system in an ice–water bath and measuring the clearing as fall in turbidity at various times. Changes were measured at 0.5-hr intervals for 2.5–3.5 hr and again after 18 hr.

Dinitrophenylation of the Isolated Grafts

Dinitrophenylation of the amino acid endgroups of the isolated grafts was used by us[9] as a technique for obtaining proof of grafting in the case of collagen-vinyl graft copolymers. This technique has been subsequently followed by Arai et al.[15] for the endgroup analysis of isolated PMMA from the graft copolymers of wool. Hence in the present paper, in continuation of the work reported earlier,[9] the absorption spectra of the dinitrophenylated grafts isolated by both acid and pronase digestion were studied.

The dinitrophenylation was carried out by two different methods. In the first method, the isolated polymer was suspended in aqueous sodium bicarbonate and treated with an alcoholic solution of 2,4-dinitrofluorobenzene(DNFB), according to the procedure adopted by Sanger.[16] In the second procedure, the isolated polymer was dissolved in benzene and treated with DNFB in the presence of triethylamine by the method of Whalley[17] as followed by Arai et al.[15] The dinitrophenylated polymer-(DNP-polymer) was precipitated by adding excess cold methanol. The DNP-polymers obtained by the two procedures were washed thoroughly with distilled water and soxhlet extracted with methanol for 12 hr. The purified samples, when subjected to thin-layer chromatography on silica gel plates as reported earlier,[9] showed only one spot which remained at the origin. No other spots corresponding to dinitrophenol or any other artifacts were observed on the plate.

The purified DNP-polymers were dissolved in ethyl acetate (4 g/l.), and the absorption spectra were measured in a Beckman DU-model spectrophotometer with the use of 1-cm quartz cells. The absorption spectra of the isolated polymers without dinitrophenylation were also measured under identical conditions. In the case of polymer isolated by pronase digestion the DNP-polymer was not readily soluble in ethyl acetate, and hence in this case it was dissolved in dichloroacetic acid(DCA).

Precipitation Turbidimetric Titrations

Collagen grafted with PMMA (sample GC_1) was dissolved in dichloroacetic acid by keeping the suspension for a few days at room temperature. The solution was then centrifuged, and the clear solution was suitably diluted with DCA to give a 0.1% solution. Turbidity measurements were made in a Klett-Summerson photoelectric colorimeter with a green filter. Different precipitants were tried, and diisopropyl ether was finally selected. A 5-ml portion of the polymer solution was taken, and the precipitant was added rapidly from a microburet with gentle hand stirring. The precipitant was added in 1-ml aliquots until within 1 ml of the commencement of precipitation. Then 0.1 ml aliquots were added until 50% precipitation, and 0.5–1.0 ml aliquots were then used until precipitation was complete. All the titration experiments were performed in a room kept at 21°C but were not otherwise thermostated.

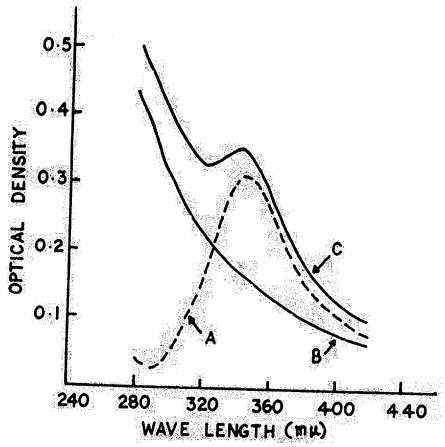

Fig. 1. Absorption spectra of (*A*)DNP–serine; (*B*) isolated polymer, (*C*) DNP-polymer in ethyl acetate (isolated by acid hydrolysis).

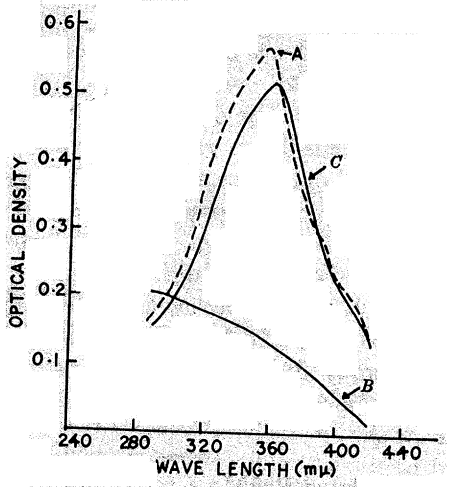

Fig. 2. Absorption spectra of (*A*) DNP–serine; (*B*) DNP–polymer in dichloroacetic acid (isolated by pronase digestion); (*C*) isolated polymer.

spectral absorption curves from 280 to 420 mμ for isolated DNP–MMA polymers are shown in Figures 1 and 2. The dinitrophenylated grafts isolated by both the acid and enzyme methods showed an absorption maximum at 343 mμ in ethyl acetate and at 360 mμ in dichloroacetic acid. DNP–serine also showed identical spectra in these two solvents. The isolated polymer without dinitrophenylation did not show any maximum in these regions. The DNP grafts isolated by the enzyme method showed more pronounced absorption maxima in the 340–360 mμ region, which indicates that the number of DNP-amino acid endgroups in these polymer chains is greater than in the case of grafts isolated by the acid hydrolysis method. This is to be expected because in the enzyme digestion method

TABLE II
Average Molecular Weight of Isolated PMMA and Number of DNP-Amino Acid Endgroups per Polymer Chain Determined by the DNP Method

Sample	Average mw of isolated grafts (by viscosity)	Number of DNP-amino acid endgroups per polymer chain	
		By sodium bicarbonate method	By triethylamine method
Collagen–PMMA graft copolymer (GC_3)[a]	0.82×10^5	0.41	0.45
Collagen-PMMA graft copolymer (GC_1)	2.075×10^6	Trace	Trace

[a]In the preparation of this sample, the ceric ion concentration used was 1×10^{-2} mole/l.

bigger peptide fragments will be attached to the polymer chain which may contain more amino groups. Table II shows the DNP-amino acid values obtained for the grafts isolated by the acid hydrolysis method. The number of amino acid endgroups calculated per polymer chain is less than unity in both the methods. The lower values obtained may be due to the inaccessibility of the amino groups to the bulky reagent. When the molecular weight of the grafted product was extremely high, the number of amino acid endgroups was so insignificant as barely to be detected by dinitrophenylation. Only trace of DNP-amino acid could be detected in these isolated grafts. In the case of grafts isolated by pronase, no attempt was made to calculate the DNP-amino groups, since in this case the polymer chains are attached to large peptide fragments.

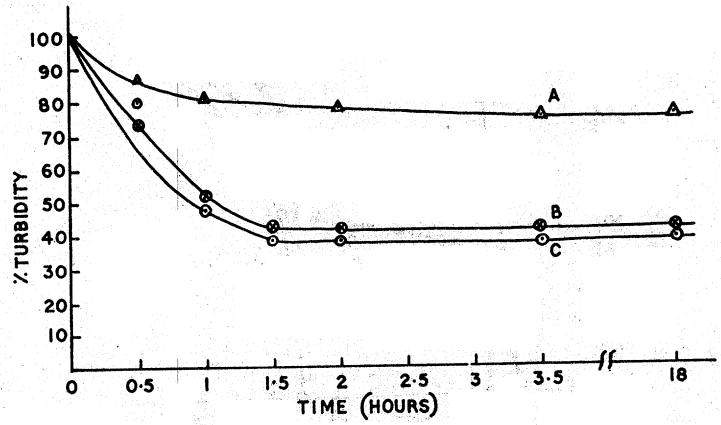

Fig. 3. Redispersion of acrylamide-grafted soluble collagen: (*A*) grafted collagen; (*B*) physical mixture of soluble collagen and polyacrylamide; (*C*) control untreated collagen.

Fibril Formation

The redispersion property of soluble collagen grafted with PAA is shown in Figure 3. In the case of the control, the reconstituted fibrils redissolved to the extent of about 60% at the end of 4 hr, whereas in the case of PAA grafted collagen the redispersion was only about 20%. These results show that the redispersion property of soluble collagen was impaired probably by attachment of the polyacrylamide(PAA) side chain to the soluble collagen molecule. Analysis of the heat-precipitated collagen after grafting also showed the presence of AA polymer in the precipitate. The physical mixture of soluble collagen and PAA, on the other hand, redissolved on cooling and behaved as untreated soluble collagen.

Precipitation Turbidimetric Titrations

The turbidimetric titration method is closely related to the method of fractional precipitation. The time-consuming operation, work-up and analysis of the fractions is here, in the words of Morey and Tamblyn,[19] replaced by "optical weighing." Even though the turbidimetric titration method is only a relative method for the determination of the molecular weight distribution, it is a simple, rapid, and reproducible method for the characterization of graft copolymers.

Curve *A* in Figure 4 represents the turbidimetric titration of a solution of collagen in DCA against diisopropyl ether. Per cent turbidity, that is, the ratio of optical density to optical density at complete precipitation, is plotted against the volume of precipitant added. Curve *B* represents the titration of a solution of PMMA, curve *C* of a physical mixture of collagen and PMMA, and curve *D* that of collagen–PMMA graft copolymer, under identical conditions. It can be seen that in curve *C* of the physical mixture, the turbidity increases rapidly on addition of 6–10 ml of diisopropyl ether and again on addition of 18–25 ml. These steps may be attributed to

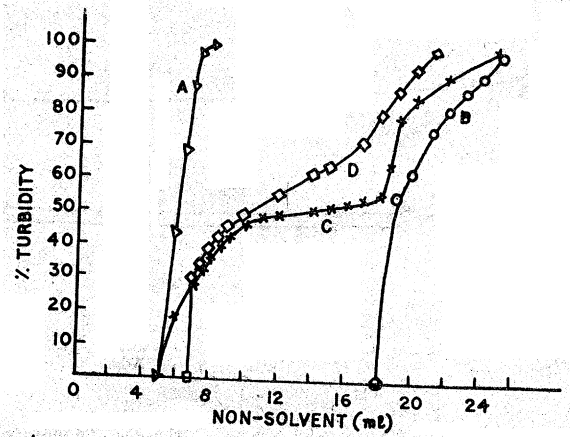

Fig. 4. Variation in turbidity with added nonsolvent: (*A*) collagen; (*B*) PMMA; (*C*) physical mixture of collagen and PMMA; (*D*) collagen–PMMA graft copolymer.

the precipitation of collagen and PMMA fractions, respectively. However, in the case of graft copolymers, the curve D is more or less continuous, and no well marked inflection was observed. This indicates that actual grafting has occurred. Generally, the chemical nature of the polymer has a much greater influence on its solubility than its molecular weight. The meager data available in the literature indicate that a graft copolymer will normally display solubility characteristics intermediate between those of the homopolymers. The solubility of collagen graft copolymer is intermediate between those of the corresponding polymers.

Viscosity in Mixed Solvents

Benoit and his co-workers[20–22] have established that the solution properties of graft copolymers are very much affected by interactions between chemically unlike sequences. If viscosity measurements are made in solvent–nonsolvent medium in which one solvent is a good solvent for both the backbone and branches, and the nonsolvent is a solvent for the branches only, repulsive interactions between the backbone and nonsolvent can be large enough to cause the formation of so-called molecular micellae.[23–25] The molecular micellae are maintained in solution by the surrounding solvated branches even though the backbone remains unsolvated.

In Figure 5 plots of percentage of benzene against the intrinsic viscosity of collagen–PMMA and collagen–PMA graft copolymers are shown. There is a slight increase in the intrinsic viscosity initially, followed by a significant decrease as the amount of benzene is increased. Protection of the backbone by solvated grafts often prevents precipitation. This may be due to the formation of the so-called polymolecular micellae. When the protection effect is not sufficient to keep the solution in molecular dispersion (as the benzene content increases) the macromolecule shrinks,

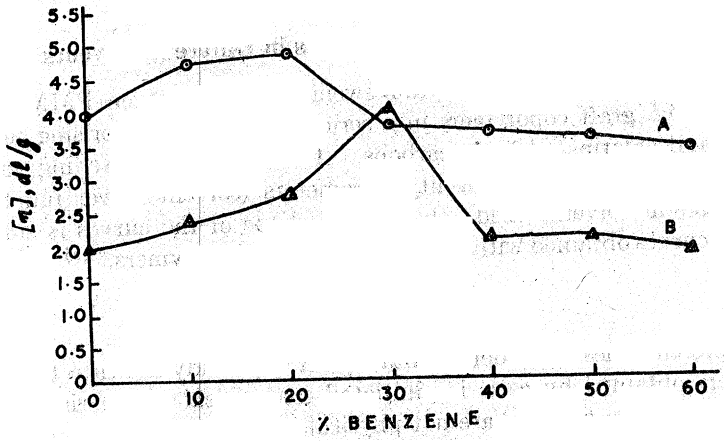

Fig. 5. Variation of intrinsic viscosities for graft copolymers of collagen as a function of solvent composition: (*A*) collagen–PMMA graft copolymer; (*B*) collagen–PMA graft copolymer.

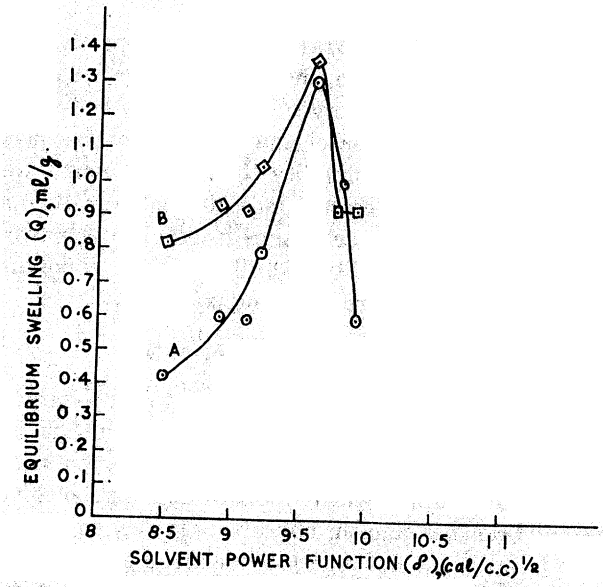

Fig. 6. Dependence of swelling of copolymers on solvent power function: (*A*) collagen–PMMA graft copolymer; (*B*) Collagen–PMA graft copolymer.

with the result that the intrinsic viscosity decreases very rapidly. The slight increase in the intrinsic viscosity observed initially may be due to an increase of the molecular dimensions of the polymer caused by changes in conformation or due to the expansions of the graft polymer chains as benzene (which is a good solvent for the side chains) is added to the solvent. Such initial expansion followed by contraction of graft copolymers in other solvents has already been reported. The shape of the curves obtained in the present study were, however, in general agreement with those of other graft copolymers obtained by other investigators.[26–28]

Equilibrium Swelling Measurements in Different Solvents

Figure 6 illustrates the equilibrium swelling of the collagen–PMMA and collagen–PMA graft copolymers in a series of solvents comprising esters, ketones, and chlorinated hydrocarbons. The equilibrium swelling values were plotted against the solubility parameters (solvent power function) of the different solvents used; the general shape of the curves is similar to that of curves obtained with other types of graft copolymers.[29,30]

CONCLUSIONS

The different lines of evidence obtained in this study indicate that the copolymers obtained by ceric ion-initiated free-radical polymerization of vinyl monomers on collagen are not physical mixtures of collagen and the homopolymer. Indications that our products are true grafts are provided by the turbidimetric titration behavior of the grafts, their general behavior

with respect to swelling in different solvents, the intrinsic viscosity of the copolymers in mixed solvents and by the detection of the DNP-amino acid endgroups in the isolated grafts. The ceric ion method of grafting vinyl monomers onto various substrates is known to have the advantage that little or no homopolymer is formed. In the present study, an attempt was also made to remove all occluded and loosely bound homopolymers by exhaustive and prolonged extraction with the appropriate solvents for the homopolymers. Hence the apparent per cent grafting shown in Table I can be taken for all practical purposes as the true per cent grafting.

The infrared spectra of the isolated grafts by acid and enzymatic hydrolysis of the grafted products and the electron microscopic studies of grafted collagen fibrils and ultrathin sections of fibrils are reported in another paper under publication.

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